Degradation of tyrosine aminotransferase (TAT) via the ubiquitin–proteasome pathway

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Abstract Most of the known cellular substrates of the ubiquitin system are short-lived growth regulators and transcriptional activators. Very few enzymes involved in intermediary metabolism have been shown to be targeted by the system. In a reconstituted cell-free system, we show that tyrosine aminotransferase (TAT), a key enzyme involved in amino acid metabolism, is conjugated and degraded in an ATP- and ubiquitin-dependent manner. Degradation of ubiquitin–TAT adducts requires, in addition to the 26S proteasome, a novel, yet unidentified, factor. TAT can be protected from degradation by association with its coenzyme pyridoxal phosphate. To examine the potential role of the ubiquitin system in regulating the stability of the enzyme in vivo, we show that cell extracts derived from livers of animals in which TAT was induced, display a corollary increase in the formation of specific TAT–ubiquitin adducts.

Key words: Tyrosine aminotransferase; Ubiquitin; Proteolysis

1. Introduction

Tyrosine aminotransferase (TAT; EC 2.6.1.5) is the rate-limiting enzyme in tyrosine catabolism. It prevents accumulation of toxic levels of tyrosine and provides ketogenic and gluconeogenetic substrates when supply of glucose to cells is limited and amino acids serve as a major source of energy. The enzyme, a homodimer composed of two identical 454 amino acid residue polypeptide chains, is expressed only in the liver and is extremely unstable with a half-life of 1–2 h [1].

Activity of TAT is highly regulated by changes in its rate of synthesis and degradation. The enzyme is strongly induced by glucocorticoids that increase the rate of transcription [2]. cAMP also increases TAT activity by increasing the level of the transcript. However, cAMP can partially induce the enzyme under conditions of complete inhibition of mRNA synthesis, suggesting that its effect is mediated also by a post-transcriptional mechanism, such as stabilization of the mRNA or inhibition of degradation [3]. Insulin, that also induces activity of the enzyme, has only a small and transient effect on the level of TAT transcript, and appears to act via a post-transcriptional mechanism. It exerts its full effect in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a potent inhibitor of mRNA synthesis, conditions under which the effect of glucocorticoids is completely blocked [3]. Spencer and colleagues have shown that insulin selectively slows the degradation rate of the enzyme [4]. Detailed structural analysis revealed that short sequences near both ends of TAT are necessary for its rapid degradation. The required basic sequence near the N-terminus (33-R**KK**GR**K**AR-40) contains three Lys residues that can potentially serve as ubiquitination sites. The second sequence (450-EECDK-454) is located at the very C-terminus of the molecule and is part of a PEST (Pro, Glu, Ser, Thr) domain implicated in targeting short-lived proteins for degradation [5]. Another factor that probably plays a role in determining the stability of the enzyme is its coenzyme, pyridoxal phosphate (PLP). The presence of PLP during purification of the enzyme is essential for the maintenance of its activity [6]. It has been shown that the relative rate of PLP dissociation from different transaminases aligns with the shortness of the half-lives of these enzymes [7]. In all these studies, the cellular system and the mechanism(s) involved in the degradation of TAT have not been identified, although it has been recently shown that many short-lived and regulatory cellular proteins are targeted via the ubiquitin pathway.

The ubiquitin-dependent pathway plays an important role in the degradation of many short-lived regulatory proteins such as cyclins and CDK, tumor suppressors, oncoproteins, and transcriptional activators and their inhibitors. Degradation of a protein via the ubiquitin system involves two successive steps: conjugation of multiple molecules of ubiquitin to the target protein and degradation of the tagged molecule by the 26S proteasome complex. Selection of proteins for degradation can be mediated via primary (constitutive) or secondary signals such as post-translational modifications or association with ancillary proteins or cofactors (reviewed in refs. [8] and [9]). So far, only a single enzyme, fructose-1,6-biphosphatase, that is involved intermediary metabolism, has been described as a substrate of the ubiquitin system [10,11]. This cytoplasmic key gluconeogenetic enzyme is subject to catabolite inactivation in the yeast *S. cerevisiae*. Addition of glucose to yeast grown on non-fermentable carbon source leads to rapid degradation of the enzyme that follows phosphorylation-dependent inactivation of its catalytic activity.

Here we show that TAT is degraded by the ubiquitin system in vitro and is protected by its coenzyme PLP. In liver cells, there is a strong correlation between the level of TAT and the ability of the cells to catalyze formation of TAT–ubiquitin adducts that serve as proteolytic intermediates.

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Abbreviations: CM, carboxymethyl; DAB, 3,3′-diaminobenzidine; DEAE, diethylaminoethyl; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; E1, ubiquitin-activating enzyme; E2, ubiquitin-carrier protein or ubiquitin-conjugating enzyme ; E3, ubiquitin–protein ligase; α-KG, α-ketoglutarate; α-LA, α-lactalbumin; PLP, pyridoxal phosphate; TAT, tyrosine aminotransferase; TCA, trichloroacetic acid; UBC, ubiquitin-conjugating enzyme

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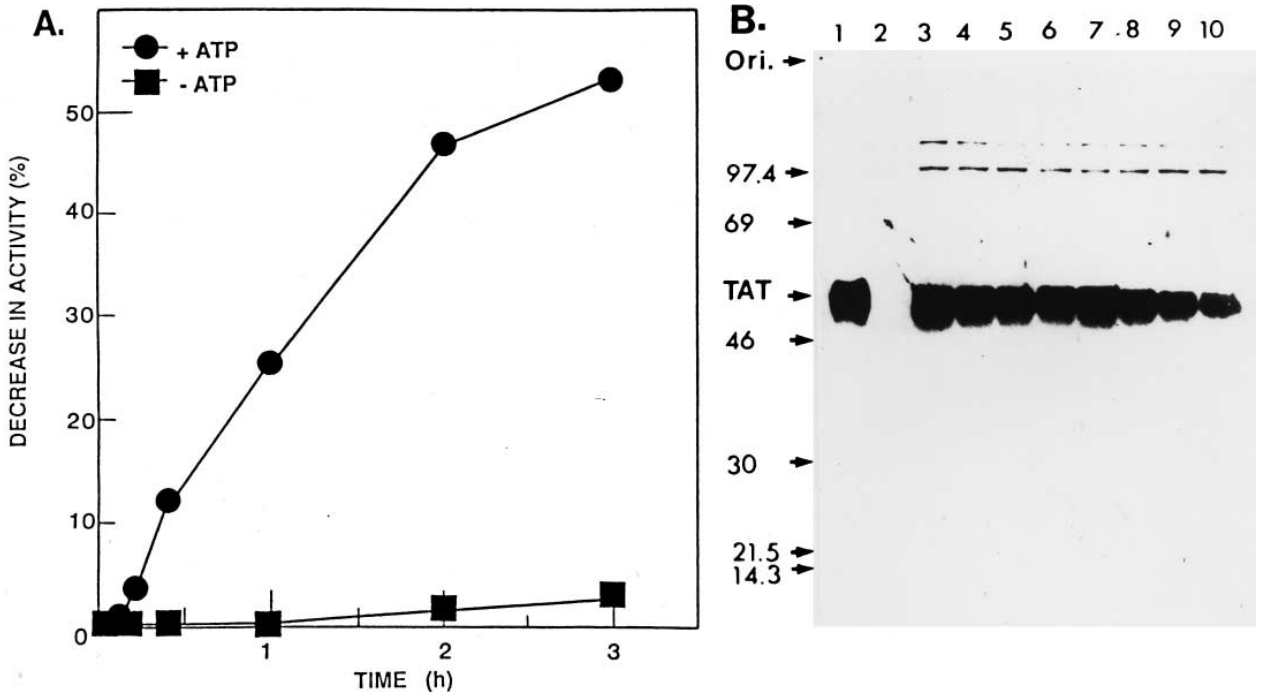

Fig. 1. A: Time- and ATP-dependent decrease in TAT activity. TAT (70 ng) was incubated in a reaction mixture containing rabbit reticulocyte lysate in the presence or absence of ATP for the indicated time periods and the changes in activity of the enzyme were monitored as described under Section 2. Results are expressed as decrease in activity. B: Time- and ATP-dependent degradation of TAT. 21 ng TAT was incubated in reticulocyte lysate and degradation was monitored via Western blot analysis following SDS-PAGE as described under Section 2. The resolved reaction contained 3.5 ng of the enzyme. Lane 1: 3.5 ng of untreated, native TAT. Lane 2: reaction mixture incubated in the absence of TAT. Lanes 3–6 and 7–10: reaction mixtures incubated in the absence or presence of ATP, respectively, for 0, 30, 60, and 120 min. MW markers are indicated. Ori., origin of gel.

2. Materials and methods

2.1. Materials

Sepharose-immobilized protein G was from Pharmacia. Antibodies were from Bio Makor. Materials for SDS-PAGE were from Bio-Rad. Dexamethasone phosphate, PLP, L-Tyr, α-KG, L-Glu, α-LA, and fine chemicals and buffers were purchased from Sigma or Boehringer. DEAE (DE52) and CM (CM52) cellulose were from Whatman. Nitrocellulose paper was from Schleicher and Scheull. All other chemicals were of high analytical grade.

2.2. Methods

2.2.1. Preparation of purified TAT and anti-TAT serum. TAT was purified from rat livers as described [6]. To remove PLP from the enzyme and the proteolytic extracts, samples were dialysed against a buffer containing 125 mM KPi (pH 7.6), 2 mM L-glutamate, and 1 mM DTT. Anti-TAT serum was a gift from Dr. Christopher Pogson (Wellcome Research Laboratories, Kent, UK). Immunoglobulins were further purified by $(NH_4)_2SO_4$ (40%) precipitation (40% saturation).

2.2.2. Assay of TAT activity. TAT activity was assayed as described [12]. To monitor activity following proteolysis in reticulocyte lysate, it was necessary to remove hemoglobin that also absorbs at 331 nm, the wavelength at which the TAT reaction product, *p*-hydroxyphenylpyruvate, absorbs. Following incubation, 500 μl of 50% CM cellulose slurry in a buffer containing 50 mM MES (pH 6.35), 1 mg/ml BSA, 1 mM EDTA, 1 mM DTT, and 0.1 mM PLP were added to the 50 μl reaction mixture and activity was determined in the supernatant. Degradation was calculated from the decrease in activity as compared to a reaction mixture that did not contain ATP or that was incubated on ice.

2.2.3. Determination and iodination of proteins. Protein was determined using the Bradford method [13]. Proteins were iodinated using the chloramine-T method as described [14]

2.2.4. Preparation and fractionation of rabbit reticulocytes lysate. Lysate was prepared from reticulocyte-rich rabbit blood and was resolved by anion exchange chromatography over DEAE-cellulose into unadsorbed material (Fraction I) and high-salt eluate (Fraction II) as described [14].

2.2.5. Conjugation and degradation assays. Degradation and conjugation assays of TAT were performed essentially as described [15,16] with slight modifications. Reaction mixtures contained in a final volume of 50 μl: 35 μl reticulocyte lysate (or PLP-depleted lysate; see above) or 200 μg protein of Fraction II. When indicated, Fraction I (25 μl) and/or ubiquitin (5 μg) were added. Reaction mixtures were resolved via SDS-PAGE (10%), and the proteins blotted onto Nitrocellulose paper as described [17]. Following blocking of non-specific binding sites, the paper was incubated successively at 4°C in the presence of sheep anti-TAT or sheep pre-immune IgG (50 μg/ml), rabbit anti-sheep IgG (10 μg/ml), and HRP-conjugated goat anti-rabbit IgG (1 unit HRP/ml). Proteins were visualized using the DAB [18] or the ECL methods. Degradation of ^{125}I-labeled proteins was monitored by measuring the release of radioactivity into TCA-soluble fraction [14].

To demonstrate a correlation between the level of the enzyme in vivo and the level the corresponding conjugating activity, labeled ubiquitin was incubated in the presence of rat liver cytosolic extracts in which TAT was induced by starvation and dexamethasone [6]. Reaction mixtures were similar to those described above except that Fraction II derived from cytosolic extracts was used as a source for the conjugating enzymes, TAT was endogenous, and the reaction contained instead of unlabeled ubiquitin ^{125}I-labeled ubiquitin (∼1 μg; ∼1 000 000 cpm). Following incubation, the reaction mixture was brought to 0.5 ml by the addition of a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 150 mM NaCl, 1% Triton X-100, 2 mM PMSF, and 20 μg/ml aprotinin. Pre-immune sheep IgG was added (25 μg; preclearing) and removed by the addition of immobilized Protein G (10 μl of 50% suspension). Specific anti-TAT IgG or pre-immune sheep IgG (25 μg) were added, and the IgG was adsorbed onto immobilized Protein G. The beads were collected and washed. Following addition of sample buffer, the immunoprecipitate was resolved via 10% SDS-PAGE, and the gel dried and exposed to a film.

2.2.6. Preparation of rat liver extracts. Liver extracts that contain all the conjugating enzymes were prepared from untreated or starved and dexamethasone-treated rats as described [6]. Following centrifugations at 9000×*g* for 15 min and at 100 000×*g* for 60 min, the supernatant was collected and frozen at −70°C.

3. Results

3.1. Purified TAT and assay of activity

Active TAT was purified to almost homogeneity (not shown). To measure enzyme activity in reticulocyte lysates, it was necessary to remove hemoglobin. That because hemoglobin and the enzymatic reaction product of TAT, *p*-hydroxyphenylpyruvate, absorb strongly at 331 nm. We utilized the difference in the p*I*'s between the two proteins to adsorb hemoglobin to the cation exchange resin CM cellulose at pH 6.35. At this pH hemoglobin adsorbs whereas more than 85% of the activity of TAT remains soluble (not shown).

3.2. Degradation of TAT in rabbit reticulocyte lysate requires ATP

In order to establish a cell-free system that degrades TAT and to study the potential role of the ubiquitin pathway in the process, we monitored the disappearance of TAT activity in rabbit reticulocyte lysate. Since activation of ubiquitin as well as the assembly and activity of the 26S proteasome require ATP, it was important to monitor the requirement for this nucleotide in the cell-free system. As can be seen in Fig. 1A, disappearance of TAT is completely dependent upon addition of the nucleotide. ADP and GTP did not have any effect (not shown). To rule out the possibility that the incubation in the presence of ATP leads to inactivation of the enzyme and to demonstrate that the protein molecule is completely degraded, we monitored the fate of TAT by Western blot analysis. As can be seen in Fig. 1B, addition of ATP leads to a time-dependent disappearance of the antigen (lanes 7–10). In contrast, depletion of ATP stabilizes the enzyme (lanes 3–6). Interestingly, degradation of TAT does not lead to the formation of intermediate products, and proteolysis proceeds to completion.

3.3. Ubiquitin- and ATP-dependent conjugation of TAT

To demonstrate the intermediacy of ubiquitin conjugation in TAT proteolysis, we monitored the ability of the molecule to covalently tag TAT. As can be seen in Fig. 2, addition of increasing amounts of ubiquitin lead to the formation of high MW ubiquitin-TAT adducts that were not formed in the absence of energy.

3.4. ATP-, ubiquitin-, and Fraction I-dependent degradation of TAT

To examine whether ubiquitin can promote degradation of TAT, we incubated the enzyme in the presence of Fraction II. As can be seen in Table 1, addition of ubiquitin did not affect the stability of the enzyme. Thus, whereas ubiquitin can generate high MW adducts with TAT, these adducts cannot be degraded in Fraction II that contains the 26S proteasome complex. In our previous studies, we have shown that Fraction I contains the protein synthesis elongation factor EF-1α that is required for the degradation of certain *N*-α-acetylated proteins [19]. EF-1α is not required for the conjugation of these proteins, but rather for their degradation by the 26S proteasome. It should be noted that the N-terminal residue of TAT is acetylated [20]. Therefore, we set to examine the effect of Fraction I on the degradation of TAT. As can be seen in Table 1, addition of Fraction I stimulates the degradation of TAT significantly (∼3-fold). Addition of purified EF-1α did not have any effect (not shown), suggesting that a novel, yet unidentified factor, is involved in proteolysis of TAT. We monitored the effect of Fraction I on the fate of the protein moiety of TAT. As can be seen in Fig. 3, addition of ubiquitin to Fraction II leads to formation of conjugates, but not to disappearance of the enzyme molecule (lane 6). In contrast, when Fraction I is also added, TAT and its ubiquitin adducts disappear (lane 9).

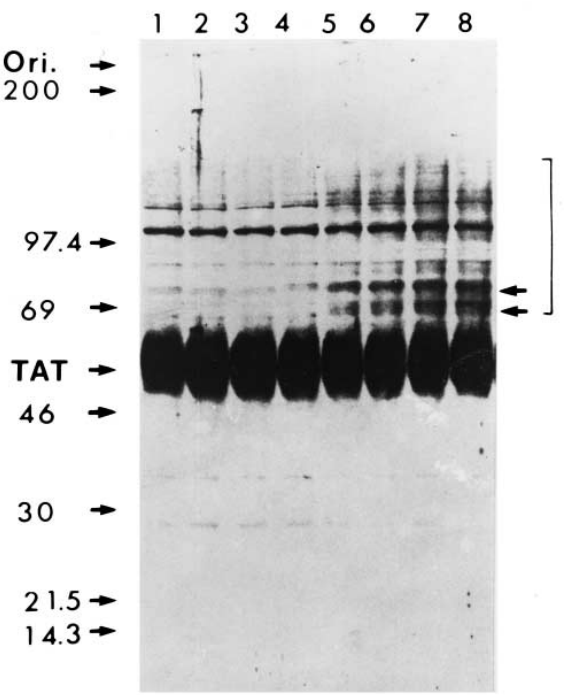

Fig. 2. Ubiquitin- and ATP-dependent conjugation of TAT. 70 ng of TAT was incubated in the presence of Fraction II in the absence (lanes 1–3) or presence (lanes 4–8) of ATP. Ubiquitin was added at 0 (lanes 1 and 4), 2 (lane 5) 4 (lanes 2 and 6) 8 (lane 7) and 16 (lanes 3 and 8) μg. Reaction mixtures (a portion containing 24 ng) were resolved and proteins visualized as described in the legend to Fig. 2. MW markers are indicated. Ori., origin of gel.

Table 1
ATP-, ubiquitin- and Fraction I-dependent degradation of TAT in the presence of rabbit reticulocyte Fraction II

Experimental conditions	Fraction II	Fraction II+ubiquitin	Fraction II+ubiquitin+Fraction I
−ATP	2.4	3.1	2.9
+ATP	4.2	8.6	24.5
Net ATP	1.8	5.5	21.6

Degradation of TAT was monitored in the different reaction mixtures as described under Section 2.

3.5. Effect of PLP on the degradation of TAT

Several lines of evidence indicate that the association between TAT and its coenzyme PLP, protects the enzyme from proteolysis (see in Section 1). To test the effect of PLP ubiquitin-mediated degradation of TAT, we depleted PLP from the TAT preparation as well as from the proteolytic extract. As can be seen in Fig. 4A, PLP specifically protects TAT, but does not have any effect on the degradation of other proteins (BSA, α-LA, and lysozyme) that do not associate with this cofactor. To demonstrate that the absence of PLP does not affect only the activity of the enzyme, we followed the effect of the cofactor on the TAT molecule. As can be seen in Fig. 4B, PLP-depleted TAT disappears completely when incubated in the absence of PLP. In contrast, addition of PLP protects the enzyme from degradation (compare lanes 3 and 6). Since the TAT preparation and the proteolytic lysates contain a small amount of PLP (final concentration in the reaction mixture is ~0.5 μM), it is clear why the degradation of the non-depleted enzyme in the untreated lysate is not complete (Fig. 1B, lane 10).

3.6. Formation of TAT–ubiquitin conjugates in the liver is proportional to the level of the enzyme

To examine the relationship between the level of the enzyme in the liver and the rate of formation of ubiquitin conjugates that serve as proteolytic intermediates, we followed the formation of these adducts in untreated livers and in livers in which the enzyme was induced (10–30 fold) following starvation and administration of dexamethasone. Labeled ubiquitin was incubated in the presence of ubiquitin-depleted liver extract and specific TAT conjugates were precipitated using anti-TAT antibody. As can be seen in Fig. 5, formation of specific conjugates is significantly higher in the extract derived from treated animals (compare lanes 3 and 4). Two independent lines of experimental evidence indicate that the high MW derivatives are indeed ubiquitin–TAT adducts. (1) They are formed only in the presence of ATP-γ-S (compare lanes 1 and 2 to lanes 3 and 4), and (2) preimmune serum fails to precipitate them (compare lanes 3 and 4 to lanes 7 and 8). To rule out the possibility that the treatment of the rats affects ubiquitin conjugation to the general population of proteins, we incubated labeled ubiquitin with extracts of livers derived from untreated, starved, and starved and dexamethasone-treated rats. Quantitative analysis reveals that there is no difference in the conjugation of ubiquitin to endogenous proteins in all three extracts (not shown).

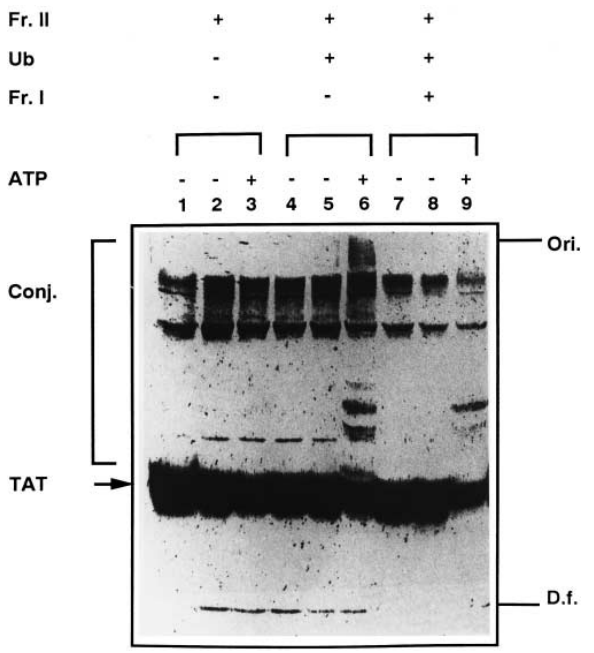

Fig. 3. Effect of Fraction I on the degradation of TAT and TAT conjugates. Reaction mixtures containing 21 ng TAT were incubated and resolved, and proteins visualized as described in the legend to Fig. 2. Lanes 1–3: reactions carried out in the presence of Fraction II. Lanes 4–6: as in Lanes 1–3, but in the presence of 5 μg ubiquitin. Lanes 7–9: as in Lanes 4–6, but in the presence of 25 μl Fraction I. Reactions were incubated in the absence (lanes 1, 2, 4, 5, 7 and 8) or presence (lanes 3, 6 and 9) of ATP. Reactions resolved in lanes 1, 4, and 7 were incubated on ice, whereas reactions resolved in lanes 2, 3, 5, 6, 8, and 9 were incubated at 37°C for 2 h. Conj., conjugates; Df., dye front; Ori., origin of gel.

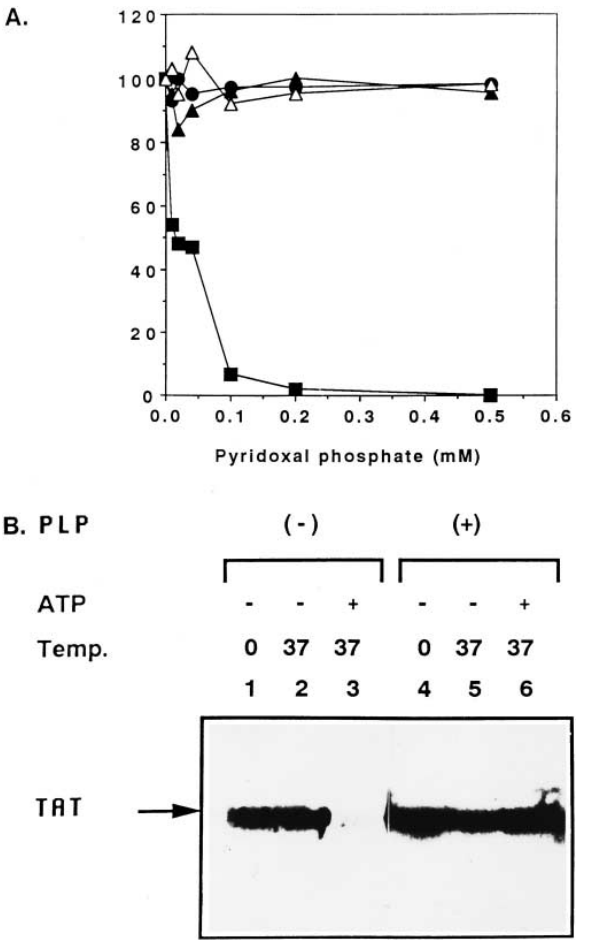

Fig. 4. A: Effect of pyridoxal phosphate on the degradation of native TAT (■), and ^{125}I-labeled BSA (●), α-LA (▲) and lysozyme (△). Degradation of TAT and the labeled proteins was monitored in a PLP-depleted reticulocyte lysate in the presence of the indicated amounts of PLP as described under Section 2. 100% degradation is the net ATP-dependent degradation. B: Effect of pyridoxal phosphate on the degradation of native TAT. 21 ng apo-TAT was incubated in the presence of TAT-depleted reticulocyte lysate in the absence (lanes 1–3) or presence (lanes 4–6) of PLP (0.5 mM), and degradation was monitored as described in the legend to Fig. 1B.

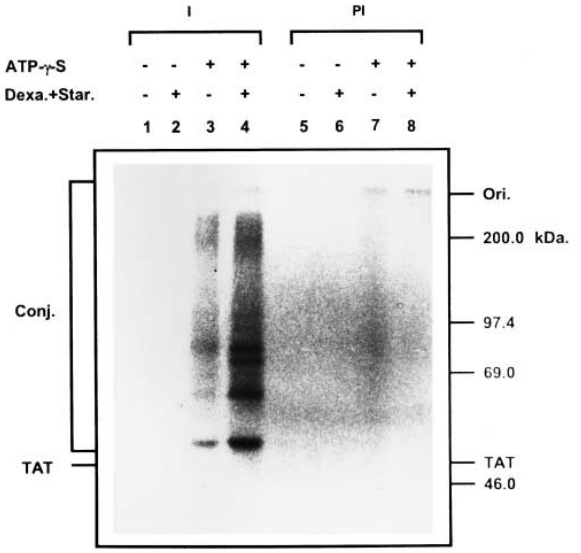

Fig. 5. Conjugation of ^{125}I-ubiquitin to TAT in liver extracts: effect of dexamethasone and starvation treatment. ^{125}I-labeled ubiquitin was incubated in the presence of rat liver Fraction II derived from untreated or starved and dexamethasone-treated rats as described under Section 2. ^{125}I-ubiquitin–TAT conjugates were immunoprecipitated by anti-TAT, resolved via SDS-PAGE, and visualized by autoradiography as described under Section 2. MW markers are indicated. Conj., conjugates; Ori., origin of gel.

4. Discussion

We have shown that the degradation of native TAT in vitro is mediated by the ubiquitin proteolytic system and the enzyme can be protected by its coenzyme, PLP. In cell extracts, the rate of formation of conjugates of ubiquitin with native endogenous TAT is proportional to the steady-state level of the enzyme, suggesting that TAT is targeted by the ubiquitin system in vivo as well.

To reconstitute activity, it was necessary to add Fraction I that contains a novel, yet unidentified factor required for the degradation of conjugates. This factor is distinct from EF-1α required for the degradation of conjugates of several *N*-α-acetylated proteins [19]. What can be the role of the factor in Fraction I? It can be, for example, an isopeptidase that trims polyubiquitin chains from the conjugates and thus rendering them susceptible to the activity of the 26S proteasome. Without this activity, the conjugates are not recognized by the protease complex. Recent evidence indicates that Fraction I indeed contains several isopeptidatic activities (unpublished results). This factor can also be a subunit or an activator of the protease that is involved in degradation of specific subset of proteins. Several activators of the 20S protease, PA28 for example, have been described recently [21], however, their function has remained elusive.

It has been suggested that many enzymes are protected by their co-factors; however, a direct role for a co-factor in the stabilization of the core protein of an enzyme from degradation by a specific cellular proteolytic system has not been demonstrated. Here we demonstrate that PLP specifically protects TAT from ubiquitin-mediated degradation. It is possible that the co-factor changes the three-dimensional structure of the enzyme and limit the accessibility of the ubiquitin–TAT ligase (E3) to a specific recognition/binding site. Alternatively, E3 binds to the protein, but the E2 cannot transfer ubiquitin to a sterically hidden specific Lys residue. Interestingly, PLP binds to a specific Lys residue [20], and it will be interesting to study whether this residue serves as a ubiquitination site. Also, it is possible that PLP sterically hinders the Lys residues in the N-terminal domain that is involved in targeting TAT for degradation (see in Section 1). It should be noted, however, that the physiological significance of PLP protection is not clear yet. Protection occurs at concentrations that exceed the K_d. Also, vitamin B6 (the precursor of PLP) deficiency does not alter the stability of the enzyme. On the other hand, a significant proportion of TAT is always found in the cell in its apo form, devoid of its co-factor [22]. Also, as mentioned in Section 1, there are strong in vitro as well as in vivo correlations between the association parameters of transaminases with PLP and their stabilities. Thus, it appears the factors that affect the association of TAT and PLP and the role of PLP in determining the stability of the enzyme in the cell are complex and need further investigation.

An important, yet unresolved problem is whether the activity of the ubiquitin system is regulated and the rate of degradation is influenced by changing physiological conditions. In any event, regulation of TAT by degradation is important, as this is the only way to remove the enzyme following induction of its synthesis.

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